PATENTANWALTSKANZLEI
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Patent application

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Method for producing a cell and/or tissue and/or disease phase specific medicament

The present invention relates to a method for producing of a cell and/or tissue and/or disease phase specific medicament suitable for the treatment of chronic inflammatory diseases.

Background of the invention

Chronic inflammatory diseases constitute an increasing medical problem area with a high socio-economic impact. These include in particular the following disease groups:

- Autoimmune diseases and rheumatic diseases (manifestations on skin, lungs, kidney, vascular system, connective tissue, musculoskeletal system, endocrine system, among other)
- Immediate-type allergic reactions and asthma
- Chronic obstructive pulmonary diseases (COPD)
- Arteriosclerosis
- Psoriasis and contact eczema
- Chronic rejection reactions after organ or bone marrow transplant

In the last decades, many of these diseases are showing a rising prevalence, not only in the industrial nations, but partly, worldwide. Thus, in Europe, North America, Japan and Australia, more than 20 % of the population already suffers from allergic diseases and asthma. Chronic obstructive pulmonary diseases are currently the fifth most common cause of death worldwide and, according to calculations by WHO, will become the third most common cause of death by the year 2020. Arteriosclerosis, with the secondary diseases myocardial infarction, stroke and peripheral arterial occlusion disease, occupy a leading position in the global morbidity and mortality statistics. Psoriasis and contact eczema are, together with neurodermatitis, the most common chronic inflammatory skin diseases.

The so far only insufficiently understood interactions between environmental factors and genetic disposition result in a subsequent defective regulation of the immune system. Here, the following common principles can be established for these different diseases:

(A) An excessive immune response against antigens, which would normally be harmless for humans, is found. These antigens can be environmental matter (e.g. allergens such as pollen, animal hairs, food, mites, chemical substances such as preservatives, dyes, cleaning products). In these cases, the patients develop an allergic reaction. In the case of, for example, active and passive cigarette smoking, chronic obstructive pulmonary diseases (COPD) occur. On the other hand, the immune system can, however, also react against the components of the own organism, recognize them as foreign and start an inflammatory reaction against them. An autoimmune disease develops in these cases. In all cases, harmless, non-toxic antigens are recognized as foreign or dangerous and an inappropriate inflammatory reaction is started.

(B) The diseases proceed in phases which include initiation, progression, i.e. progressing of the inflammatory reaction, and the associated destruction and alteration with loss in organ functionality (so-called remodelling).

(C) The diseases present patient specific subphenotypic characteristic features.

(D) Components of the innate and acquired immunity are later involved in the initiation, maintenance and in the destruction and alteration processes. Under the influence of the innate immunity (important components: antigen presenting cells with their diverse populations and the complement system), the cells of the adaptive immune system (important components: T- and B-lymphocytes) are activated and differentiated. T-cells take over central functions in the following process by differentiating into highly specialized effectors. In doing so, they activate and acquire certain effector mechanisms, including, in particular, the following functions: Production of antibodies, control of

the functionality of effector cells of the immune system (such as, for example, neutrophil, basophil, eosinophil granulocytes), feeding back on functions of the innate immune system, influencing the functionality of non-hematopoietic cells, e.g. epithelium, endothelium, connective tissue, bone and cartilage, and, in particular, neuronal cells. This amounts to a special interaction between immune and nervous system, which has led to the development of the concept of neuro-immunological interaction in chronic inflammations.

Due to the complexity and variety of the disease patterns associated with chronic inflammations, an optimal medicament for the treatment of the diseases must meet the following requirements:

(1) Diseases manifest themselves in patient specific (sub)phenotypes. Medicaments must therefore possess a high patient or case specificity.

(2) Diseases proceed in stages and phases. Medicaments must therefore possess a high stage or phase specificity.

(3) The diseases are regulated by cells of different specialization. Medicaments must therefore cause a cell specific intervention.

(4) The diseases manifest themselves in different organs and compartments. Medicaments must therefore possess a high compartment or organ specificity.

(5) Medicaments must be suitable for a long-term therapy. Immune system reactions against the medicaments must therefore be prevented.

(6) The side effect profile of the medicaments must present an acceptable medical and ethical balance between severity

index, prognosis and progress of the disease.

None of the currently available established therapies against chronic inflammations meets these criteria in an optimal way. The treatment with immunoglobulin A is known from DE 695 11 245 T2, and the inhibition of phospholipase A_2 (PLA_2) and/or coenzyme A-independent transacylase (CoA-IT) is known from DE 695 18 667 T2. For this disease, the currently established therapy concepts are centred on unspecific anti-inflammatory therapy, as well as immune suppression. Many of the applied unspecific anti-inflammatory substances, such as ibuprofen, acetylsalicylic acid and paracetamol, are either not effective enough or are afflicted with a high rate of unwanted side effects. Steroids may have, in contrast, a higher potency, but are themselves afflicted with serious side effects, such as hypertonia, diabetes and osteoporosis. New generation immune suppressing medicaments, such as, for example, cyclosporine and tacrolimus, present hepato- and nephrotoxicity.

This situation has led to the search for and the clinical testing of a plurality of new molecules intended to act more specifically on immunological and cell biological defective regulation. These include cytokines, cytokine receptors and anti-cytokines. Problems related to these new therapeutic applications include a lack of cell and organ specificity, development of unwanted immune reactions against these molecules, and poor effectiveness for different phenotypes.

In recent years, attempts are being made to use a new class of catalytic molecules, the so-called "DNAzymes" (Santoro 1997), as therapeutic agents for inactivating genes, the expression of which is the cause of diseases. DNAzymes are single stranded molecules which can, in principle, bind to complementary areas of the RNA and inactivate it through cleavage. The specific use of DNAzymes as therapeutic

agents requires, however, that the genes causing the disease, as well as their RNA, are known in detail. This is so far only the case for few diseases.

The DNAzyme described in WO 01/11023A1 binds RelA (p65) mRNA and is thus directed against the transcription factor NF-κB; WO 00/42173 discloses an EGR-1 mRNA binding DNAzyme. WO99/50452 discloses a 10-23 DNAzyme that can be used in a diagnostic method for detecting nucleic acid mutations.
None of the currently known antisense molecules and DNAzymes can be used for producing a medicament for the treatment of chronic inflammations in patients.

Object of the invention

The object of the present invention is the provision of cell and/or tissue and/or disease phase specific medicaments which lead to the functional inactivation of ribonucleic acid molecules of transcription factors and factors of signal transduction pathways, the expression of which is involved in the development of chronic inflammatory reactions and autoimmune diseases, and which are suitable for the treatment of chronic inflammatory reactions and autoimmune diseases, thus eliminating the abovementioned disadvantages of the state of the art.

It is a further object of the invention to provide a method for producing cell and/or tissue and/or disease phase specific medicaments, which identifies ribonucleic acid molecules of transcription factors and factors of signal transduction pathways, the expression of which is involved in the development of chronic inflammatory reactions and autoimmune diseases, and functionally inactivates them in target cells.
According to the present invention this object is solved by a method according to claim 1 and a medicament according to claims 16 to 18 using specific DNAzymes according to claims

10 to 15.

The advantage of the invention lies in a functional inactivation of ribonucleic acid molecules of transcription factors and factors of signal transduction pathways for differentiation and/or expression of cytokines which are involved in the development of chronic inflammatory reactions and autoimmune diseases, by means of specific DNAzymes and/or siRNA. This strategy distinguishes itself from conventional but also gene therapeutic approaches by a very high cell and/or tissue and/or disease phase specificity and selectivity, high stability of the molecules and negligible antigenicity. Optimal preconditions for a tailored long-term therapy for patients with chronic inflammatory diseases are created.

Further details and advantages of the present invention will become apparent from the following figure and description. The following is shown:

Fig. 1: schematic representation of the signal transduction during differentiation of $CD4^+$ cells to TH1 and TH2 cells, respectively (modified according to Ho I.C. and Glimcher L.H., Cell 2002; 109; S109-S120).

Fig. 2: Nucleotide sequence of the catalytic domain of the 10-23 DNAzyme and binding to a target RNA by means of Watson-Crick pairing. (R = A or G; Y = U or C, N = A, G, U or G). The arrow indicates the cleavage site on the target mRNA.

Fig. 3: Pool of specific ribonucleic acid molecules according to step b) particularly the DNAzymes hgd 1 to hgd 70 against GATA-3 and their nucleotide sequences (A=Adenine, G=Guanine, C=Cytosine, T=Thymine). Nucleotides written in uppercase mark a right and left substrate binding domain, nucleotides written in lowercase mark the central catalytic

domain of the 10-23 DNAzyme.

Fig. 4: Nucleotide sequences of human GATA-3 genes in alignment.
Sequence 1: Human GATA-3 from database no.: XM_043124.
Sequence 2: Human GATA-3 from database no.: X58072.
Sequence 3: Human GATA-3 (sequenced from plasmid pCR2.1).
Divergent bases are highlighted in grey, primer locations for GATA-3 cloning are underlined. The localisation of the DNAzyme hgd40 is pointed out with bold letters which are also highlighted in grey and underlined.
(A=Adenine, G=Guanine, C=Cytosine, T=Thymine)

Fig. 4 A: Nucleotide sequence 3 of human GATA-3 gene from figure 4, into which the individual nucleotide pairs GT and AT have been drawn (highlighted in grey), between which there are further DNAzyme cleavage sites.

Fig. 5: Gel electrophoresis showing the cleavage of a target mRNA (here GATA-3 mRNA) with specific ribonucleic acid molecules according to step b), here unmodified DNAzymes [hgd11 (lane 2), hgd13 (lane 4), hgd17 (lane 6), hgd40 (lane 8)] and modified DNAzymes [hgd11-M (lane 3), hgd13-M (lane 5), hgd17-M (lane 7), hgd40-M (lane 9)]. M designates the modified DNAzymes. Unmodified (0.25 µM) or modified DNAzymes (0.25 µM) are incubated for one hour at 37 °C with in vitro transcribed GATA-3 mRNA (0.025 µM) in a volume of 10 µl comprising the following reaction composition: 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$. The products are subsequently separated by means of gel electrophoresis. Lane 1 contains a control mRNA with no added DNAzyme. A length standard run in parallel (not shown) shows band sizes of 1000 bp, 2000 bp and 3000 bp. The arrows point at S, the band containing the substrate (here GATA-3 mRNA) and the cleavage products P1 and P2.

Fig. 6: Immunoblot with the reaction of specific ribonu-

cleic acid molecules in cells. Jurkat E6.1 cells are transfected by means of lipofection with specific ribonucleic acid molecules, here DNAzymes [hgd11-M (lane 4), hgd13-M (lane 5), hgd17-M (lane 6), hgd40-M (lane 7)]. Untreated cells (lane 1), cells only treaded with transfection medium (lane 2), and cells treated with DNAzymes (hgd11-M) with no transfection medium (lane 3) were used for control purposes. After 48 h of incubation, the solubilized proteins are separated by means of SDS-PAGE and GATA-3 (A) detected by immunoblot with specific antibodies. (Lane 4 contains cells with hgd11-M, lane 5 contains cells with hgd13-M, lane 6 contains cells with hgd17-M, lane 7 contains cells with hgd40-M.) To verify that the same protein quantities are present in each lane, immunostaining with ß-actin (B) is carried out on the same blot membrane. The length standard run in parallel (not shown) shows band sizes of 63.8 kDa, 49.5 kDa and 37.4 kDa.

Fig. 7: Pool of specific ribonucleic acid molecules according to step b) particularly the DNAzymes td 1 to td 70 against T-bet and their nucleotide sequences (A=Adenine, G=Guanine, C=Cytosine, T=Thymine).

Fig. 8: Nucleotide sequences of human T-bet genes in alignment.
Sequence 1: Human T-bet from database no.: NM_013351.
Sequence 2: Human T-bet (sequenced from pBluescript-SK).
Divergent bases are highlighted in grey, primer locations for T-bet cloning are underlined. The primer locations for the relative quantification on the LightCycler are circled. The localisation of the DNAzymes td54 and td69 is highlighted in grey and underlined at the same time, td70 is additionally highlighted in bold letters.
(A=Adenine, G=Guanine, C=Cytosine, T=Thymine)
Fig. 8 A: Nucleotide sequence 1 of human T-bet gene from figure 8, the individual nucleotide pairs GT and AT drawn therein, highlighted in grey, between which there are fur-

ther DNAzyme cleavage sites.

Fig. 9: Gel electrophoresis showing the cleavage of a target mRNA (here T-bet mRNA) with specific ribonucleic acid molecules according to step b), here modified DNAzymes [td54m (lane 3), td69m (lane 4) and td70m (lane5)]. The modified DNAzymes (0.25 µM) are incubated for 30 min at 37 °C with in vitro transcribed T-bet mRNA (0.025 µM) in a volume of 10 µl comprising the following reaction composition: 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$. The products are subsequently separated by means of gel electrophoresis. Lane M contains a 3000 base and 2000 base length standard run in parallel, lane 2 contains a control mRNA with no added DNAzyme. Arrow A points at the band with substrate (here T-bet mRNA), arrow B points at the larger cleavage product. The second cleavage product is smaller and no longer visible in this figure.

Fig. 10: Quantification on the LightCycler of T-bet and GAPDH mRNA from cells treated with DNAzymes td54 (A), td69 (B) and td70 (C). Jurkat E6.1 cells are transfected twice in a period of 24 h, either with the T-bet specific DNAzymes td54 (A), td69 (B) and td70 (C) or with nonsense DNAzymes for control (not shown). After subsequent cleaning with RNA, a reverse transcription is carried out and the obtained DNA introduced in the LightCycler. GAPDH (dashed lines) is used as internal standard. Shown are 4 measurements each of cells treated with T-bet specific DNAzymes or nonsense DNAzyme. The solid lines show the quantity of T-bet in the cells treated with T-bet specific DNAzymes, dotted lines show the quantity of T-bet in the cells treated with nonsense DNAzyme.

Fig. 11: Diagram of the relative quantification of T-bet mRNA in Jurkat E6.1 cells.

Jurkat E6.1 cells are transfected twice with T-bet specific DNAzymes td54, td69 and td70, and isolated with RNA after 48 h. After a reverse transcription, the quantity of mRNA

is determined by means of LightCycler. Nonsense DNAzyme is used as control. The relative quantification of T-bet and GAPDH mRNA is carried out according to instructions [described in the User Bulletin #2 (ABI Prism 7700 Sequence detection System User Bulletin #2 (2001). Relative quantification of gene expression. Http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf)]. Here, the quantity of T-bet mRNA from the control test with nonsense DNAzyme is set equal to 100 %.

Figure 1 shows a schematic representation, modified according to Ho I.C. and Glimcher L.H., Cell 2002; 109; S109-S120, of the dynamics of the signal transduction during differentiation of $CD4^+$ cells to TH1 and TH2 cells, respectively. Stimulation via the T cell receptor through the respective peptide-MHC complex induces the clonal expansion and programmed differentiation of $CD4^+$ T lymphocytes to T helper (TH)1 or TH2 cells. Discrimination between these two sub-types takes place on the basis of their cytokine profiles. TH1 cells produce interferon-y (INFy), interleukin 2 (IL-2) and tumour necrosis factor-ß, whereas TH2 cells secrete IL-4, IL-5, IL-9 and IL-13. Bacterial and viral infections induce an immune response that is dominated by TH1 cells. On the other hand, TH2 cells regulate the production of IgE against parasites. TH1 and TH2 cells are in equilibrium with each other. The destruction of this equilibrium causes diseases, an excessive TH1 cell response being associated with autoimmune diseases, while an increased TH2 cell response is the origin of allergic diseases.

It is known that TH1 cytokines are involved in the pathogenesis of autoimmune diseases such as, for example, autoimmune uveitis, experimental allergic encephalomyelitis, type 1 diabetes mellitus or Crohn's disease, while TH2 cytokines (IL-4, IL-5, IL-13 or IL-9) take part in the development of chronic inflammatory airway diseases such as, for example, airway eosinophilia, mucus hypersecretion and air-

way hyperreactivity. The basis for these diseases are pathophysiological changes during the production of characteristic cytokines by antigen specific TH cells. For instance, transgenic mice which constitutively overexpress the TH2 cytokines IL-4, IL-5, IL-13 or IL-9 in the airway epithelia, show typical allergic inflammatory reactions. In the animal model, TH2 cell subpopulations in the lung and airways induce in TH2 cells the characteristic symptoms of bronchial asthma .

Surprisingly, it was found that transcription factors and factors of signal transduction pathways for differentiation and/or expression of cytokines which are involved in the development of chronic inflammatory reactions and autoimmune diseases, such as, for example, the TH1 cell specific transcription factor T-bet and the TH2 cell specific transcription factor GATA-3, are ideally suited for the cell and/or tissue specific treatment of chronic inflammations or autoimmune diseases.

The TH1 cell specific transcription factor T-bet is, above all, responsible for the differentiation of naive CD^+ T cells to TH1 cells. Its expression is controlled by signal transduction pathways of the T cell receptor (TCR) and through INFy receptor/STAT1 T-bet transactivates the endogenous INFy gene and induces the production of INFy. Furthermore, it induces the up-regulation of the protein expression of IL-12Rß2 chains and leads to chromatin remodelling of individual INFy alleles. The in vivo function of T-bet has been confirmed on knock-out mice (T-bet). Although T-bet deficient mice present a normal lymphocyte development, $CD4^+$ T cells from these mice produce no INFy, neither when stimulated with anti-CD3/CD28 nor with PMA/ionomycine. T-bet deficient mice display no immune response to a *L. major* infection, the amount of TH2 cytokines increases.

The function of T-bet in mucosal T cells during the devel-

opment of inflammatory bowel diseases is known. Investigations on the animal model show a worsening of colitis in reconstituted SCID (Severe Combined Immunodeficiency) mice after retroviral transduction of T-bet in $CD4^+CD26L^+$ T cells, while, conversely, the transfer of T-bet deficient T cells does not lead to an induction of colitis.

The transcription factor T-bet specifically induces the development of TH1 cells and controls the production of INFy in these cells. The inhibition of T-bet shifts the balance between TH1 and TH2 cells towards the TH2 cells.

The TH2 cell specific transcription factor GATA-3 is, above all, responsible for the differentiation of naive $CD4^+$ T cells to TH2 cells.

Here, the TH2 cell differentiation is controlled mainly by two signal transmission paths, the T cell receptor (TCR) path and the IL-4 receptor path. Signals forwarded by the TCR activate the TH2 cell specific transcription factors c-Maf and GATA-3 as well as transcription factors NFAT and AP-1. The activation of the IL-4 receptor leads to the binding of STAT6 to the cytoplasmatic domain of the IL-4 receptor, where it is phosphorylated by Jak1 and Jak3 kinases. The phosphorylation leads to dimerisation and translocation of STAT6 into the nucleus, where STAT6 activates the transcription of GATA-3 and other genes.
GATA-3 is a zinc finger transcription factor which, according to "Representational Difference Analysis" (RDA) and studies on transcriptional regulation of IL-5, is expressed exclusively in mature TH2 cells and not in TH1 cells. Further transcription factors that play a role in the differentiation to TH1 cells or TH2 cells, respectively, and are involved in the development of chronic inflammatory and autoimmune diseases present an expression which is different in a target cell than compared to a control cell expression and are, according to the present invention, also

used in the design of specific DNAzymes and/or siRNA for therapeutic application in chronic inflammatory diseases.

- STAT4, STAT5a and STAT1 (signal transducer and activator of transcription)
- c-Rel
- CREB2 (cAMP response element-binding protein 2)
- ATF-2, ATF-2
- Hlx
- IRF-1 (interferon regulatory factor-1)
- c-Maf
- NFAT (Nuclear factor of activated T cells)
- NIP45 (NF-AT interacting Protein 45)
- AP1 (Activator Protein 1)
- Mel-18
- SKAT-2 (SCAN box, KRAB domain associated with a Th2 phenotype)
- CTLA-4 (Cytolytic T lymphocyte-associated antigen 4)

Further factors of the signal transduction pathways that play a role in the differentiation and/or expression of cytokines and are involved in the development of chronic inflammatory and autoimmune diseases exhibit an expression in the target cell that differentiates itself from a control cell expression and are, according to the present invention, also used in the design of specific DNAzymes and/or siRNA for therapeutic application in chronic inflammatory diseases.

- Src kinase
- Tec kinase
  - Rlk (Txk in humans)
  - Itk
  - Tec

- RIBP (Rlk/ltk-binding protein)
- PLCy (Phospholipase Cy1)
- MAP kinase (Mitogen-activated protein kinase)

ERK
JNK
P38

- MKK (MAP kinase kinase)
  MKK1
  MKK2
  MKK3
  MKK4
  MKK6
  MKK7
- Rac2
- GADD45 (Growth arrest and DNA damage gene 45)
  GADD45ß
  GADD45γ
- SOCS (Suppressors of cytokine signalling)
  CIS (Cytokine-induced SH2 protein)
  SOCS1
  SOCS2
  SOCS3
- JAK (Janus kinase)
  JAK1
  JAK3
- NIP45 (NF-AT interacting Protein 45)

According to the present invention, a cell and/or tissue and/or disease phase specific medicament is provided which is suitable for the treatment of chronic inflammatory diseases.
The medicament acts preferably on the intervention points of the complex cascade of immunological and cell biological defective regulations forming the basis for chronic inflammatory reactions and autoimmune diseases. Particularly preferably, these are intervention points in the regulation of the differentiation of the transcription factors involved, such as, for example, the TH2 cell specific transcription factor GATA-3 or the TH1 cell specific transcription factor T-bet. The therapeutic effect achieved is based

on a functional inactivation of ribonucleic acid molecules by means of specific DNAzymes and/or SiRNA. This strategy offers a series of advantages compared to conventional, but also gene therapeutic approaches: highest specificity and selectivity, high stability of the molecules and a negligible antigenicity. Optimal preconditions for a tailored long-term therapy for patients with chronic inflammatory diseases are created.

According to the present invention a method for producing of a cell and/or tissue and/or disease phase specific medicament is provided comprising the steps of:

a) Identification of ribonucleic acid molecules the expression of which is different in a target cell than compared to a control cell expression

b) Design of specific ribonucleic acid molecules which bind to ribonucleic acid molecules from step a) and functionally inactivate them

c) Introduction of specific ribonucleic acid molecules from step b) into target cells

d) Formulation of the specific ribonucleic acid molecules from step b) and/or a target cell from step c) into a medicament

In the sense of the present invention, the term "cell and/or tissue and/or disease phase specific" means that the medicament produced by means of the method according to the present invention is substantially only effective in a certain type of cell (target cell) and/or in certain tissues or organs and/or in certain phases of the disease, and has a negligible influence on other cells (control cells) tissues or organs. Preferably the medicament is effective in at least 2/3 of the target cells, more preferably in at

least 80 % and most preferably on at least 98 % of the target cells. It is further preferred that the medicament is effective in no more than 10 % of the control cells, more preferred in no more than 5 % and most preferred in < 1 % of the control cells.

In the present invention the term "Identification of ribonucleic acid molecules the expression of which is different in a target cell than compared to a control cell expression" comprises the following points:

i) Target cells are cells in tissues and organs which are known to lead to the development of a disease, contribute thereto or aggravate that disease, which support the processes sustaining the disease, contribute thereto or compound those processes, or which lead to late effects of a disease, contribute thereto or aggravate those effects. They include, for example, cells which present certain transcription factors, secrete specific hormones, cytokines and growth factors, or cells with typical surface receptors.

ii) The target cells can be isolated, for example, by means of technologies which are based on the binding of specific antibodies. Magnetic Beads, obtainable from the companies Miltenyi (Macs-System), Dynal (DynaBeads) or BD-Bioscience (iMAG) are used here. Alternatively, this takes place through cell purification by means of fluorescent labelled antibodies on cell sorters, for example from the company Cytomation (MOFLO) or BD-Bioscience (FACS-Vantage). The purity of the target cells is preferably at least 80 %, more preferably at least 95 % and most preferably at least 99 %.

iii) Methods for isolating RNA are described, e.g. in Sambrook and Russell, Molecular Cloning, A Laboratory Manual, 3. edition, Cold Spring Harbor Laboratory (2001), New York und Ausubel et al., Current Protocols in Molecular Biology,

John Wiley & Sons (1998), New York. In addition, it is possible for the average person skilled in the art, to use commercially available kits for RNA isolation (silica technology) e.g. the RNeasy kit from the company Qiagen. It is further preferable to purify mRNA directly from the target cells by using commercial kits, for example from the companies Qiagen (Oligotex mRNA kit), Promega (PolyATract mRNA Isolation System) or Miltenyi (mRNAdirect).

iv) Identification of incrementally different mRNAs, i.e. mRNAs with an expression in the target cell that is higher than the control cell, is conducted, for example, with commercially obtained gene chips (e.g. MWG, CLON-TECH)] or with a filter hybridization method (e.g. Unigene), according to the manufacturer's instructions. Alternatively, differential mRNAs are produced by subtractive hybridization of cDNA which had previously been created from the mRNA through RT reaction . Included in these methods known to the person skilled in the art are, for example, the SSH method (Clontech) or the RDA method. A further preferred application form includes the combination of chip technology and subtractive hybridization. Identification of the differentially expressed genes is carried out using chip technology with the help of commercially available programs, e.g. with the Vector Xpression program from the company InforMax. When using subtractive hybridization, after isolation of the differentially expressed genes by means of conventional methods known to the person skilled in the art, such as cloning and subsequent sequencing (see e.g. Sambrook and Russell, Molecular Cloning, A Laboratory Manual, 3. edition, Cold Spring Harbor Laboratory (2001), New York and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons (1998), New York), a sequence alignment in a data base such as, for example, GenBank (www.ncbi.nlm.nih.gov) is carried out. The expression within the target cell is different compared to the expression in a control cell. In an embodiment of the method ac-

cording to the present invention the expression in the target cell is higher than the expression in a control cell, preferably at least by a factor 1.5. In a particularly preferred embodiment the expression in the target cell is higher than the expression in a control cell by at least a factor 5, and in a most preferred embodiment the expression is only detectable in the target cell but not in the control cell.

In the sense of the present invention, the term "Design of ribonucleic acid molecules which bind to ribonucleic acid molecules from step a) and functionally inactivate them" comprises the use of RNA inactivating DNA enzymes (DNAzymes) and/or small interfering RNA (siRNA), which functionally inactivates ribonucleic acid molecules.

According to the present invention the term DNAzymes hereby comprises DNA molecules that specifically identify and cleave the target sequence of the nucleic acid, both DNA and RNA.

The "10-23" model represents a general DNAzyme model. DNAzymes of the10-23 model - also called "10-23 DNAzymes" - possess a catalytic domain of 15 deoxyribonucleic acids, which is flanked by two substrate binding domains. The length of the substrate binding domains is variable, they can either be of the same or of different length. In a preferred embodiment, the substrate binding domains are between 6 and 14 nucleotides long. In a particularly preferred embodiment the substrate binding domains are fully complementary to the region flanking the cleavage site. To bind to and cleave the target RNA, the DNAzyme must not necessarily have to be fully complementary. In vitro investigations show that DNAzymes of the 10-23 type cleave the target RNA at purine-pyrimidine sequences.

To use DNAzymes in the treatment of diseases, preferably,

the DNAzymes are stabilized as well as possible against degradation within the body (in the blood, in the intracellular environment, etc.). In a preferred embodiment, a 3'-3' inversion is introduced at one or more ends of the DNAzyme. The term 3'-3' inversion denotes a covalent phosphate bond between the 3'carbon atoms of the terminal nucleotide and the adjacent nucleotide. This type of bond is located, as opposed to the normal phosphate bond, between the 3' and 5' carbon atoms of consecutive nucleotides. Accordingly, it is preferred that the nucleotide on the 3' end is inverse to the of the 3' end of the substrate binding domain adjoining the catalytic domain. In addition to the inversions, DNAzymes can comprise modified nucleotides or nucleotide compounds. Modified nucleotides contain, e.g.. N3'-P5' phosphoramidate compounds, 2'-O-methyl substitutions and peptide nucleic acid compounds. Their production is known to the person skilled in the art.

Although the potential DNAzyme cleavage sites occur ubiquitously, they are often blocked by the secondary RNA structure and are thus inaccessible to the DNAzymes. For this reason, only those DNAzymes with freely accessible cleavage sites are selected from a DNAzyme pool. These selected DNAzymes are active, cleave the target mRNA, thus functionally inactivating it. The efficiency of the mRNA cleavage by the individual DNAzymes is shown either by individual testing of each DNAzyme or by coupled testing of multiple DNAzymes in "multiplex assays" (described, e.g. in Cairns et al., 1999).

According to the present invention, the term siRNA comprises 21-23 base long RNA molecules which lead to a specific degradation of the complementary target mRNAs, both in vitro and in vivo. On the basis of the available literature (e.g. http://www.mpibpc.gwdg.de/abteilungen/100/105/index.html), the person skilled in the art is familiar with the produc-

tion of siRNA molecules starting from the target mRNA sequence. The probability that among three selected siRNA molecules at least one of them is highly active (inhibition of the target RNA by at least 80 %), is stated as being at least 70 % in the literature. From a pool of siRNA molecules, only those are selected which lead to a specific degeneration of the complementary target mRNA, both in vitro and in vivo.

In the sense of the present invention, the term "introduction of the specific ribonucleic acid molecules from step b) into target cells" comprises the transfection into the target cells of vectors, particularly plasmids, cosmids, viruses or bacteriophages, which contain the previously described specific ribonucleic acid molecules according to the present invention. Preferably, the vectors are suited for transformation of animal and human cells and allow the integration of the ribonucleic acid molecules according to the present invention. Transfection methods such as, for example, lipofection with DMRIE-C from the company Invitrogen are known to the person skilled in the art from the literature. In principle, liposomal vectors are also suited therefor. The target molecules are transcription factors, cells secreting hormones, cytokines and growth factors, but also cells carrying the expressed receptors on the surface. The control cells in the sense of the invention are healthy cells from the target tissue, cells of the same type from other compartments of the same patient or also from healthy individuals.

Cultivation of the target cell is carried out in culture media adapted to the requirements of the target cell in regard to pH value, temperature, salt concentration, antibiotics, vitamins, trace elements and ventilation. The term patient relates equally to humans and vertebrates. The medicament can therefore be used in both human and veterinary medicine.

The term "Formulation of the specific ribonucleic acid molecules from step b) or a target cell from step c) into a medicament" comprises pharmaceutically acceptable compositions containing modifications and "prodrugs", as long as they do not trigger excessive toxicity, irritations or allergic reactions in the patient after conducting a reliable medical assessment. The term "prodrug" relates to compounds which are transformed to improve their absorption, for example, by hydrolysis in the blood.

Preferably, the formulation permits the specific ribonucleic acid molecules to be administered to the patient in form of a pharmaceutically acceptable composition, either orally, rectally, parenterally, intravenously, intramuscularly or subcutaneously, intracisternally, intravaginally, intraperitoneally, intrathecally, intravascularly, locally (powder, ointment or drops) or in spray form.

Dosing forms for local administration of the medicament of this invention comprise ointments, powders, sprays or inhalation means. The active component is admixed under sterile conditions, depending on the requirements, with a physiologically acceptable carrier substance and possible preservatives, buffers or propellants.

The dosing method is determined by the treating physician in accordance with the clinical factors. It is known to the person skilled in the art that the dosing method is dependent on different factors, such as, for example, body size, weight, body surface area, age, sex or the general health of the patient, but also on the specific substance to be administered, the duration and type of administration, and on other medicaments which are possibly administered in parallel.

The medicament produced with the method according to the

present invention possesses a high patient, disease, stage and phase specificity. It causes a cell specific intervention and is specific for compartments and organs. No or only very limited reactions of the immune system develop against the medicament and the side effect profile is commensurate with severity index, prognosis and progression of the disease.

The medicament can be used in the therapy of all disease groups associated with chronic inflammations, such as, for example, autoimmune diseases, rheumatic diseases (manifestations on skin, lungs, kidney, vascular system, connective tissue, musculoskeletal system, endocrine system, among other), immediate-type allergic reactions and asthma, chronic obstructive pulmonary diseases (COPD), arteriosclerosis, psoriasis and contact eczema, and also in the therapy of chronic rejection reactions after organ or bone marrow transplant.

Execution examples

Example 1: GATA-3

a) Identification of ribonucleic acid molecules the expression of which is different in a target cell than compared to a control cell expression

i) The naive $CD4^+$ cells responsible for the development of chronic inflammatory reactions are used as target cells.

ii) The $CD4^+$ target cells are isolated using magnetic beads (from Miltenyi (Macs System) Dynal (DynaBeads) or BD-Bioscience (iMAG)), alternatively on cell sorters by means of fluorescent labelled antibodies, e.g. from the companies Cytomation (MOFLO) or BD Bioscience (FACSVantage).

iii) RNA isolation is carried out according to standard methods; see Sambrook and Russell, Molecular Cloning, A Laboratory Manual, 3. edition, Cold Spring Harbor Laboratory (2001), New York und Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons (1998), New York.

Alternatively, an RNeasy kit from the company Qiagen is used, or the mRNA is isolated directly from CD4+ target cells using the Oligotex mRNA kit from the company Qiagen, according to manufacturer's instructions.

iv) Identification of incrementally different mRNAs, i.e. mRNAs with a higher expression in the target cell than the control cell, is conducted with gene chips (e.g. MWG, CLONTECH), and the identification of the differentially expressed genes is carried out by means of the Vector Xpression program from the company InforMax.

Filter hybridization method (e.g. Unigene), according to manufacturer's instructions. The isolation of the differen-

tially expressed genes is followed by cloning, sequencing (according to standard procedures, see e.g. Sambrook and Russell, Molecular Cloning, A Laboratory Manual, 3. edition, Cold Spring Harbor Laboratory (2001), and sequence alignment in the gene data base (www.ncbi.nlm.nih.gov). The expression of GATA-3 is different in the target cell (TH2 cell) in comparison with the expression in the control cell (for example Th0 cell).

b) Design of specific ribonucleic acid molecules which bind to ribonucleic acid molecules from step a) and functionally inactivate them

Figure 3 shows the pool hgd 1 to hgd 70 of specific DNAzymes according to the present invention against GATA-3 mRNA. The DNAzymes have a total length of 33 nucleotides, with the central catalytic domain corresponding to 15 nucleotides (in lowercase letters) of the catalytic domain of the known 10-23 DNAzymes (figure 2). This catalytic domain is flanked by two right and left substrate binding domains (in uppercase letters), each comprising 9 nucleotides. The nucleotide sequence of the right and left substrate binding domain is different and varies for the DNAzymes hgd 1 to hgd 70, so that a different specific bond takes place by means of Watson-Crick pairing to the GATA-3 mRNA.

Figure 2 shows the general model for binding the 10-23 DNAzymes to an arbitrary target RNA, labelled N, wherein the arrow points to the cleavage site on the target mRNA. Although DNAzymes can cleave the target mRNA at every purine-pyrimidine sequence, it is known from literature that purine-uracil bonds are cleaved more effectively than purine-cytosine bonds. For this reason, DNAzymes which cleave at purine-uracil bonds are preferably constructed. The model shown in figure 2 can be applied, in terms of its operating principle, to the binding of the DNAzymes hgd1 to hgd70 to GATA-3 mRNA..

The DNAzymes hgd1 to hgd70 are used unmodified for in vitro tests and modified for tests on cell cultures (purchased through the company Eurogentec). The following modifications were applied for stabilization and protection:

1) A stabilizing inverse thymidine on the 3' end.
2) a FAM label on the 5' end to assess the transfection efficiency of the cells by means of FACS analysis.

Testing the DNAzymes in vitro requires GATA-3 mRNA that has been produced by in vitro transcription. The individual steps are as follows:

- RNA isolation from human EDTA whole blood by means of a QIAamp RNA Blood Mini Kit (Qiagen, Germany), according to manufacturer's instructions.

- reverse transcription with the primers:

Forward primer GGCGCCGTCTTGATACTTT

Reverse primer CCGAAAATTGAGAGAGAAGGAA, with amplification of a 2731 nucleotide long PCR product (JumpStart Accu Taq DNA polymerase, Sigma).

PCR conditions: Initial denaturation (96 °C, 30 sec.), amplification for 40 cycles (94 °C, 15 sec.; 48 °C, 30 sec.; 68 °C, 3 min.), final extension (68 °C, 30 min.).

The PCR product is cloned into the plasmid pCR2.1 (Invitrogen) using standard procedures and sequenced for verification. Production of GATA-3 mRNA is carried out after linearization of the GATA-3 containing plasmid pCR2.1 by cleaving with the restriction enzyme Spe I through in vitro transcription according to the manufacturer's instructions (Ambion). GATA-3 mRNA is present with a length of a total

of 2876 nucleotides.

Figure 4 shows the known nucleotide sequences of human GATA-3 genes obtained from database entries [PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide)], wherein divergent bases are highlighted in grey. Sequence 1: Human GATA-3 from database no.: XM_043124, sequence 2: Human GATA-3 from database no.: X58072, sequence 3: Human GATA-3 (isolated from plasmid pCR2.1).

The GATA-3 mRNA sequences differentiate themselves from each other by the length of the 3' untranslated or 5' untranslated ends. To obtain the exact complete sequence of the mRNA, the mRNA sequences of the entries no.: XM_043124 and X58072 are used for the primer selection. The primer locations for cloning GATA-3 are highlighted in figure 4 by underlining. Figure 4 also shows an alignment of the nucleic acid sequence of GATA-3 obtained from the data base (sequence 1 and 2) and the nucleotide sequence (sequence 3) sequenced from plasmid pCR2.1. It shows that the sequences are not totally identical, with some bases being different. The nucleic acid sequence 3 of GATA-3 in figure 4 forms, according to the present invention, the basis for the construction of DNAzymes against GATA-3 mRNA.

Figure 4 A shows the nucleotide sequence of sequence 3 of the human GATA-3 gene from figure 4 and, drawn therein as grey highlight, two nucleotides GT or AT, respectively, between which there are further potential cleavage sites for DNAzymes.

The in vitro cleavage experiments of GATA-3 mRNA with the DNAzymes (hgd1-hgd70) are conducted in a volume of 10 µl comprising the following reaction composition: 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.25 µM DNAzyme and 0.025 µM GATA-3 mRNA transcribed in vitro (at a substrate to DNAzyme ratio of 1:10). The reactions are incubated at 37

°C for the times indicated for each case. The reaction is stopped by adding formamide and EDTA containing RNA Sample Loading Buffer (Sigma). The denatured samples are separated on 1.3 % TAE agarose gels and analyzed in the UV transilluminator.

Figure 5 shows as the result of the gel electrophoresis the cleavage of the GATA-3 target mRNA with unmodified DNAzymes [hgd11 (lane 2), hgd13 (lane 4), hgd17 (lane 6), hgd40 (lane 8)] and modified DNAzymes [hgd11-M (lane 3), hgd13-M (lane 5), hgd17-M (lane 7), hgd40-M (lane 9)]. Lane 1 contains a control mRNA with no added DNAzyme. The modified DNAzymes are characterized with an additional M. A length standard run in parallel (not shown) shows band sizes of 1000 bp, 2000 bp and 3000 bp. The arrows point at S, the band containing the substrate (here GATA-3 mRNA) and the cleavage products P1 and P2.

The comparison between all 70 DNAzymes shows that hgd11, hgd13, hgd17 and hgd40 are particularly active, the modifications lowering the effectiveness of the DNAzyme hgd11, hgd13 und hgd17, but not the effectiveness of the DNAzyme hgd40.

The following table shows the classification of the DNAzyme hgd 1 to hgd 70 against GATA-3 mRNA in 4 groups. This classification is conducted on the basis of in vitro activity tests of the DNAzymes against GATA-3 mRNA. Group 1: high cleavage activity, group 2: average cleavage activity, group 3: weak cleavage activity, and group 4: no measurable cleavage activity.

Group	**Hgd**	**Activity against GATA-3**
1	11, 13, 17, 40	High cleavage activity

2	10, 12, 16, 18, 23, 31, 36, 37, 39, 52, 57, 58, 63, 70	Average cleavage activity
3	22, 24, 25, 34, 35, 41, 42, 43, 45, 46, 47, 48, 49, 50, 54, 55, 56, 57	Weak cleavage activity
4	1, 2, 3, 4, 5, 6, 7, 8, 9, 14, 15, 19, 20, 21, 26, 27, 28, 29, 30, 32, 33, 38, 44, 51, 53, 59, 60, 61, 62, 64, 65, 66, 67, 68, 69	No cleavage activity

c) Introduction of specific ribonucleic acid molecules from step b) into target cells

The highly active DNAzymes hgd11, hgd13, hgd17 and hgd40 are used in target cells with and without the described modifications.

For this, Jurkat E6.1 cells (human acute T cell leukemia cells) are cultivated in the RPMI medium with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 % FKS at 37 °C in a humidified 5 % CO_2 atmosphere. The transfections are carried out in 6-well plates. For this, $2x10^6$ Jurkat E6.1 cells are introduced in an Opti-MEM I cell culture medium (Invitrogen) and transfected by means of DMRIE-C (Invitrogen) with the modified DNAzymes (0.3 µM) (according to manufacturer's instructions of the company Invitrogen). After a 10 hour incubation in the incubator under the above-mentioned conditions, the RPMI medium (containing the additions indicated above) is added and the incubation continued for a further 14 hours. The cells are washed with Opti-MEM medium and subsequently transfected again following the protocol described above. The transfection efficiency is assessed after each transfection by means of FACS analysis.

The activity of the DNAzymes is subsequently verified by detecting the GATA-3 protein quantity on the Western blot (see figure 6).

For Western blot analyses the cytoplasmic proteins and the core proteins are processed separately by means of a protein extraction kit in accordance with manufacturer's instructions (Pierce). The protein concentration is determined with the BCA kit (Pierce). The separation of 30 µg protein in each case is carried out by means of denaturing gel electrophoresis in 10 % SDS polyacrylamide gels. The proteins are subsequently blotted on nitrocellulose membranes according to standard procedures. The membranes are blocked with 5 % skim milk powder in PBS (with 0.01 % Tween 20) and subsequently incubated at room temperature with mouse anti-GATA-3 antibodies (Santa Cruz) (1:500) and following this with HRP-coupled mouse anti-rabbit antibodies (BD Biosciences) (1:2000) for one hour, respectively. The proteins are visualized by means of chemiluminescence. Variations in the protein quantity applied is controlled by parallel detection of beta-actin on the blots. For that, GATA-3 is detected on the nitrocellulose membrane first. The same membrane is subsequently left over night in a humid chamber. After washing with PBS twice, the detection of ß-actin takes place through immunostaining with specific antibodies (mouse anti-human beta-actin antibody (Sigma)).

Figure 6 shows the result of the immunoblot with the resulting activity of the DNAzymes in cells. Jurkat E6.1 cells are transfected by means of lipofection with DNAzymes (lane 4=hgd11-M, lane 5=hgd13-M, lane 6=hgd17-M, lane 7=hgd40-M). Untreated cells (lane 1), cells only treaded with transfection medium (lane 2), and cells treated with DNAzymes and no transfection medium (lane 3) were used for control purposes. After 48 h of incubation, the solubilized proteins are separated by means of SDS-PAGE and GATA-3 (A) detected by immunoblot with specific antibodies. To confirm

that the same quantity of protein is being used on each lane, immunostaining with ß-actin (B) is carried out on the same blot membrane. A length standard run in parallel (not shown) shows band sizes of 63.8 kDa, 49.5 kDa and 37.4 kDa.

The results show that the DNAzymes hgd11, hgd13 and hgd17 are not active in vivo, while the DNAzyme hgd40 inhibits the GATA-3 expression also in vivo. The specific in vivo inhibition of the GATA-3 expression by the DNAzyme hdg40 hence provides an effective therapeutic tool for the treatment of chronic inflammatory diseases.

d) Formulation of the specific ribonucleic acid molecules from step b) and/or a target cell from step c) into a medicament

The analysis of different DNAzymes with a substrate domain specific to GATA-3 shows that the DNAzyme hgd40 specifically inhibits the GATA-3 expression in vivo and is suitable as specific ribonucleic acid for the production of a cell and/or tissue and/or disease phase specific medicament. For this, hgd40 (5'-GTGGATGGAggctagctacaacgaGTCTTGGAG) or cells transfected with hgd40 are provided in a pharmaceutical composition with a pharmaceutically acceptable carrier for example liposomes or biodegradable polymers.

Alternatively to the DNAzymes, the use of siRNA is proposed for the specific inhibition of the GATA-3 expression and for the production of a cell and/or tissue and/or disease phase specific medicament. Preferably siRNA is used for the inhibition of mouse and human GATA-3. The production of siRNA is known to the person skilled in the art and described in the literature. Examples for siRNA sequences are given below:

Source	Nucleic acid sequences
Mouse GATA-3	Sense strand: CAUCGAUGGUCAAGGCAACdTdT Antisense strand: GUUGCCUUGACCAUCGAUGdTdT
Human GATA-3 sequence 1	Sense strand: CAUCGACGGUCAAGGCAACdTdT Antisense strand: GUUGCCUUGACCGUCGAUGdTdT
Human GATA-3 sequence 2	Sense strand: AAGAGUGCCUCAAGUACCAdTdT Antisense strand: UGGUACUUGAGGCACUCUUdTdT
Human GATA-3 sequence 3	Sense strand: AGCUUCACAAUAUUAACAGdTdT Antisense strand: CUGUUAAUAUUGUGAAGCUdTdT
Human GATA-3 sequence 4	Sense strand: UGACUCACUGGAGGACUUCdTdT Antisense strand: GAAGUCCUCCAGUGAGUCAdTdT

Example 2: DNAzyme against T-bet

a) Identification of ribonucleic acid molecules the expression of which is different in a target cell than compared to a control cell expression

The identification takes place according to the procedure described above. The expression of T-bet is different in the target cell (Th1 cell) in comparison with the expression in a control cell (Th0 cell).

b) Design of specific ribonucleic acid molecules which bind to ribonucleic acid molecules from step a) and functionally inactivate them

The identification of cleavage sites for cleavage of T-bet is carried out as described for GATA-3.

Figure 7 shows the pool td1-td78 of specific DNAzymes ac-

cording to the present invention against T-bet mRNA. The DNAzymes have a total length of 33 nucleotides, with the central catalytic domain corresponding to 15 nucleotides (in lowercase letters) of the catalytic domain of the known 10-23 DNAzyme (figure 2). This catalytic domain is flanked by two right and left substrate binding domains (in uppercase letters), each comprising 9 nucleotides. The nucleotide sequence of the right and left substrate binding domain is different and varies for the DNAzymes td1 to td78, so that a different specific bond takes place by means of Watson-Crick pairing to the T-bet mRNA.

Since it is known from literature that DNAzymes cleave the target mRNA at purine-uracil more effectively than at purine-cytosine bonds, DNAzymes which cleave at purine-uracil bonds are preferably constructed.

The model shown in figure 2 can be applied, in terms of its operating principle, to the binding of the DNAzymes td1 to td78 to T-bet mRNA.

The DNAzymes td1 to td78 are used unmodified for in vitro tests and with the modifications described for GATA-3 for tests on cell cultures.

To present the cleavage properties of the DNAzymes and the functional inactivation of the target mRNA of the T-bet mRNA, in vitro transcription of the T-bet mRNA from human EDTA whole blood is carried out by means of a QIAamp RNA Blood Mini Kit (Qiagen, Germany)', according to manufacturer's instructions.

Figure 8 shows the nucleotide sequence of human T-bet, as obtained from the database entries [PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide)] no.: NM_013351, sequence 1.

The reverse transcription takes place with the forward primer CGGCCCGCTGGAGAGGAAGC and reverse primer CACACACCCA-CACACAACC in accordance with standard procedures (Thermo-Script from Invitrogen), with amplification of a 2450 nucleotide long PCR product. This PCR product is cloned into the pBluescript-SK (Stratagene) using standard procedures and sequenced for verification.

Figure 8 shows a comparison between the nucleic acid sequence of T-bet no.: NM_013351 (sequence 1) and the sequenced sequence (sequence 2). It shows that the sequences are not totally identical, with individual bases being interchanged. The nucleic acid sequence 2 of T-bet in figure 8 forms, according to the present invention, the basis for the construction of DNAzymes against T-bet mRNA.

Figure 8 A shows the nucleotide sequence of sequence 1 of the human T-bet gene from figure 8 and, drawn therein with grey highlight, two nucleotides GT or AT, respectively, between which there are further potential cleavage sites for DNAzymes.

Production of T-bet mRNA is carried out after linearization of the T-bet containing plasmid pBluescript-SK by cleaving with the restriction enzyme Xba I (Fermentas) and through in vitro transcription according to the manufacturer's instructions (Ambion). T-bet mRNA is present with a length of a total of 2550 nucleotides.

The in vitro cleavage experiments of T-bet mRNA with the DNAzymen (td1 to td78) are conducted and analyzed in accordance with the descriptions to GATA-3. Figure 9 shows as the result of the gel electrophoresis the cleavage of the T-bet target mRNA with modified DNAzymes [td54-M (lane 3), td69-M (lane 4), td70-M (lane 5)]. Lane 2 contains a control T-bet mRNA with no added DNAzyme. A length standard run in parallel (lane M) shows band sizes of 2000 bp and

3000 bp. The arrows point at A, the band containing the substrate (here T-bet mRNA) and B, one of the two cleavage products (the other cleavage product is not shown in this figure).

The comparison between all 78 DNAzymes shows that td54, td69 and td70 are particularly active, the modifications not decreasing the effectiveness of the DNAzyme.

The following table shows the classification of the DNAzyme td 1 to td 78 against t-bet-3 mRNA in 4 groups. This classification is conducted on the basis of in vitro activity tests of the DNAzymes against t-bet mRNA. Group 1: high cleavage activity, group 2: average cleavage activity, group 3: weak cleavage activity, and group 4: no measurable cleavage activity.

Group	**Td**	**Activity against t-bet mRNA**
1	54, 69, 70	High cleavage activity
2	21, 24, 28, 29, 30, 45, 71, 72, 77, 78	Average cleavage activity
3	13, 19, 22, 23, 25, 27, 31, 32, 44, 46, 47, 48, 50, 51, 53, 55, 56, 57, 58, 60, 61, 62, 65, 67, 68, 73, 74, 75	Weak cleavage activity
4	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 20, 26, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 49, 52, 59, 63, 64, 66, 76	No cleavage activity

c) Introduction of specific ribonucleic acid molecules from step b) into target cells

The DNAzymes td54, td69 and td70 are used in target cells with and without the described modifications. The data on the transfection of Jurkat E6.1 cells corresponds that on the execution example GATA-3. After transfection of Jurkat E6.1 cells the quantity of T-bet mRNA relative to GAPDH mRNA expression is determined quantitatively by means of real-time PCR (LightCycler, Röche) to obtain information on the in vitro effectiveness of the DNAzymes.

For LightCycler analyses the RNA from the Jurkat E6.1 cells is purified by means of RNeasy Mini Kit (Qiagen, Germany) and subsequently normalized photometrically. After reverse transcription with SuperScript II (Gibco) in accordance with manufacturer's instructions follows the quantitative analysis of the T-bet mRNA and GAPDH mRNA in the LightCycler. The total volume for the PCR is of 20µl, containing 1µl DNA, 1µl (0.5µM) for each the sense and the antisense primer, as well as 10µl QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany). The PCR primers used for T-bet are: Sense 5'-CCCACCATGTCCTACTACCG-3'; Antisense 5'-GCAATCTCAGTCCACACCAA-3'. The PCR primers used for GAPDH are: Sense 5'-CCCACCATGTCCTACTACCG-3'; Antisense 5'-GCAATCTCAGTCCACACCAA-3'. The PCR conditions are: Denaturation (15 min 95 °C), amplification (15 sec 95 °C, 25 sec 59 °C, 25 sec 72 °C for 50 cycles) then final extension 2 min 72 °C. The following melting curve is generated as follows: 0 sec 95 °C, 15 sec 60 °C then increase the temperature to 97 °C in 0.2 °C increments, simultaneously measuring the fluorescence. The melting curve is used for internal control since all PCR products have a specific melting temperature.

SYBR Green is a fluorescent dye (included in the QuantiTect SYBR Green PCR Master Mix) that binds double stranded DNA. When the DNA is doubled during the extension, SYBR Green binds to it generating a bond dependent fluorescence signal which is detected by the LightCycler at the end of every

extension. The higher the quantity of initial material, the earlier a significant increase in the fluorescence will be detected. The LightCycler software provides a graphical representation of the collected fluorescence intensities against the cycles.

Figure 10 shows LightCycler amplification curves of T-bet mRNA and GAPDH mRNA after treatment of Jurkat E6.1 cells with the DNAzymes td54m, td69m and td70m in comparison to those treated with nonsense-DNAzyme. The individual crossing point (Ct), defined as the PCR cycle at which the fluorescence first distinguishes itself significantly from the background fluorescence, is determined manually with the fit point method of the LightCycler software. The relative quantification of T-bet mRNA and GAPDH mRNA in cells treated with DNAzymes compared with cells treated with nonsense DNAzyme is carried out according to the instructions described in the User Bulletin #2 (ABI Prism 7700 Sequence detection System User Bulletin #2 (2001) Relative quantification of gene expression http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). Here, the quantity of T-bet mRNA from the control test is set equal to 100 %. The data from the relative quantification are represented graphically in figure 11.

Compared to nonsense DNAzyme treatment, it is shown that the td69m DNAzyme leads to a suppression of 81.3 % and the td70m DNAzyme to a suppression of 81.0 %, while the td54m DNAzyme has no suppressive effect on T-bet mRNA.

This means that the td54m DNAzyme is not active in vivo, while td69m and td70m DNAzymes inactivate the mRNA of T-bet also in the cellular environment. The specific in vivo reduction of the T-bet mRNA by the DNAzyme td69m and td70m hence provides an effective therapeutic tool for the treatment of chronic inflammatory diseases.

d) Formulation of the specific ribonucleic acid molecules from step b) and/or a target cell from step c) into a medicament

The analysis of different DNAzymes with a substrate domain specific to T-bet shows that DNAzymes td69 and td70 specifically inhibit the T-bet expression in vivo and are suitable as specific ribonucleic acid for the production of a cell and/or tissue and/or disease phase specific medicament.

For this, td69 (GGCAATGAAggctagctacaacgaTGGGTTTCT) or td70 (TCACGGCAAggctagctacaacgaGAACTGGGT) or cells transfected with td69m or td70, respectively, are provided in a pharmaceutical composition with a pharmaceutically acceptable carrier for example liposomes or biodegradable polymers.

Alternatively to the DNAzymes, the use of siRNA is proposed for the specific inhibition of the T-bet expression and for the production of a cell and/or tissue and/or disease phase specific medicament. Preferably this is siRNA for inhibiting human T-bet. The production of siRNA is known to the person skilled in the art and described in the literature. The following is an example for siRNA sequences:

Source	**Nucleic acid sequences**
Human T-bet	Sense strand: UCAGCACCAGACAGAGAUGdTdT Antisense strand: CAUCUCUGUCUGGUGCUGAdTdT

It is evident to the person skilled in the art that with the teachings of the present invention specific DNAzymes and siRNAs can also be easily produced as medicament for chronic inflammatory diseases and autoimmune diseases, which are directed against further transcription factors that play a role in the differentiation to TH1 or TH2

cells, respectively, for example STAT4, STAT5a, STAT1, c-Rel, CREB2, ATF-2, Hlx, IRF-1, c-Maf, NFAT, NIP45, AP1, Mel-18, SKAT-2, CTLA-4 or which are directed against further factors of the signal transduction pathways for differentiation and/or expression of cytokines, for example Src kinase, Tec kinase, Rlk (Txk in humans), ltk, *Tec*, RIBP, PLCy, MAP kinase, ERK, JNK, P38, MKK, MKK1, MKK2, MKK3, MKK4, MKK6, MKK7, Rac2, GADD45, GADD45ß, GADD45y, SOCS, CIS, SOCS1, SOCS2, SOCS3, JAK, JAK1, JAK3, NIP45.

These Proteins present an expression which is higher in a target cell when compared to the expression in a control cell.